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Characterization of 6-phosphofructo-2-kinase from foetal-rat liver

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Foetal and adult liver 6-phosphofructo-2-kinase (PFK-2) were purified by identical protocols. The native molecular masses of both enzymes were determined by gel filtration and were 89.1 and 100.0 kDa respectively. No differences were found in SDS/PAGE in 10%-acrylamide gel (55 kDa per subunit). The kinetic properties displayed by both enzymes were similar, except for the sensitivity to inhibition by *sn*-glycerol 3-phosphate. Foetal PFK-2 was a good substrate for phosphorylation by cyclic AMP-dependent protein kinase and protein kinase C, whereas the adult enzyme was phosphorylated only by cyclic AMP-dependent protein kinase. However, the phosphorylation affected only the kinetic properties of the adult enzyme, suggesting the presence in both enzymes of different sites of phosphorylation by cyclic AMP-dependent protein kinase. These differences in primary structure were consistent with the distinct chromatographic profiles of the phosphopeptides after digestion of the protein with CNBr. Western-blot analysis with antibodies specific for the *N*-terminal region of the liver-type PFK-2 poorly recognized the foetal enzyme, suggesting that both enzymes differ at least in the *N*-terminal sequence.

INTRODUCTION

Transition from the foetal to the neonatal period is accompanied by important changes in liver carbohydrate metabolism. These changes are induced by hormonal and nutritional factors [1] and by modifications in the expression pattern of enzymes and tissue-specific isoenzymes [2–4].

One of the relevant factors in the control of the glycolytic and gluconeogenic fluxes in liver is the change in fructose 2,6-bisphosphate (Fru-2,6-P_2) concentration [5]. Foetal hepatocytes contain a form of 6-phosphofructo-2-kinase (PFK-2) that exhibits marked differences from the adult form in its regulatory properties, mainly the lack of glucagon-dependent inactivation of the enzyme, which occurs for the adult tissue [6]. Accordingly, incubation of foetal PFK-2 with the catalytic subunit of the cyclic AMP-dependent protein kinase did not produce changes in the enzyme activity. Using cultured hepatocyes from 22-day foetuses we showed that incubation with glucagon for periods longer than 45 min elicited the appearance of the glucagon-responsive enzyme [7]. Moreover, a PFK-2 that behaved like the adult enzyme was detected 3 h after birth as a result of the hypoglycaemia and glucagonaemia of this period [7]. In addition, exposure of foetal hepatocytes to phorbol myristate acetate increased the PFK-2 activity and Fru-2,6-P_2 concentration, whereas this response was absent in adult cells [8]. These data strongly suggest the existence of a different isoenzyme of PFK-2 in the foetal period that exhibits some kinetic properties similar to those reported for the bovine heart or rat hepatoma-tumour-cell (HTC) enzymes [9–11].

There is clear evidence for the existence of at least two PFK-2/fructose-2,6-bisphosphatase (FBPase-2) isoenzymes: the liver (L) and the muscle (M) isoenzyme, which are the products of one gene by alternative use of two promoters. There are two mRNAs for this bifunctional enzyme: one mRNA is 2.1 kb long and encodes the L isoenzyme, and the other mRNA is 1.9 kb long and it encodes a protein that displays properties of the M isoenzyme [12,13]. A third 6.8 or 4 kb mRNA typical of cardiac tissue has been reported [14,15]. In rat hepatoma cells, the PFK-2/FBPase-2 has kinetic, antigenic and regulatory properties that differ from those found in liver. It has been suggested that HTC-cell PFK-2 is the foetal form of the enzyme [11,16].

In the present paper, we report further differences between adult and foetal liver PFK-2/FBPase-2 regarding molecular mass, phosphorylation by cyclic AMP-dependent protein kinase and protein kinase C, immunoreactivity and a different peptide composition as deduced after CNBr cleavage.

EXPERIMENTAL

Materials

Pregnant Albino Wistar rats (300–350 g) fed on a standard laboratory diet were killed for the experiments between 09:00 and 10:00 h. Gestational age was confirmed by standard criteria [17]. Newborn rats were delivered by caesarean section in the morning of day 22 of gestation. Livers were removed and stored in liquid N_2.

Substrates, coenzymes and enzymes were obtained from Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO, U.S.A). Standard analytical-grade laboratory reagents were purchased from Merck (Darmstadt, Germany). Electrophoresis reagents were from Bio-Rad (Richmond, CA, U.S.A.). Radiochemicals were purchased from Amersham International (Amersham, Bucks, U.K.).

Enzyme purification

PFK-2 was purified from adult and foetal rat liver (300–400 g of tissue) by the same procedure [18]. Livers were homogenized with an Ultra-Turrax instrument in 4 vol. (v/w) of buffer A [20 mM-potassium phosphate, 1 mM-EDTA, 2 mM-dithiothreitol (DTT), 100 mM-KCl, 0.5 mM-phenylmethanesulphonyl fluoride, 0.1 mM-fructose 6-phosphate, pH 7.4 at 4 °C]. After centrifugation (10000 *g* for 20 min), the supernatants were filtered through glass wool and fractionated with poly(ethylene glycol) 7000. The 6–15%-poly(ethylene glycol) pellet fractions were resuspended in one-fourth of the homogenate volume, in buffer A. The fractions were then applied to a column (5 cm × 25 cm) of DE52 DEAE-cellulose equilibrated with buffer A. After washing the column with buffer A until the eluate was free of protein

Abbreviations used: Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (EC 2.7.1.105); DTT, dithiothreitol.

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(monitored by the A_{280}), PFK-2 was eluted with a linear gradient of KCl (in buffer A, 100–600 mM-KCl). Fractions containing PFK-2 activity were pooled and applied to a column (2.5 cm × 15 cm) of Blue Sepharose CL-6B equilibrated with buffer B [15 mM-Hepes, 2 mM-DTT, 1 mM-EDTA, 1 mM-potassium phosphate, 100 mM-KCl, 0.1 mM-fructose 6-phosphate, 0.5 mM-phenylmethanesulphonyl fluoride, 20% (v/v) glycerol at pH 7.4]. The column was washed with buffer B, followed by buffer B containing 400 mM-KCl (buffer C), until the protein concentration in the eluate was negligible. PFK-2 was eluted (at 2.5 mM) with a linear gradient of 0–15 mM-MgATP in buffer C. The PFK-2 enriched fractions were pooled, dialysed and concentrated by freeze-drying. The enzymes were resuspended in buffer D (10 mM-Hepes, 1 mM-DTT, 1 mM-potassium phosphate, 0.1 mM-fructose 6-phosphate, 5% glycerol, at pH 7.4) and stored frozen at −80 °C. Samples of the frozen stored enzyme were thawed and further purified by gel filtration through a column (2.6 cm × 87 cm) of Sephacryl S-200 equilibrated with 10 mM-potassium phosphate/1 mM-DTT/100 mM-NaCl/2% glycerol, pH 7.4. The yield of the enzyme purification was about 20%. The purity of the preparations was determined by SDS/PAGE in 10%-acrylamide gels. Proteins were detected by Coomassie Blue [19].

To determine the molecular mass of PFK-2 from adult or foetal liver, the purified enzymes (25 μg) were analysed by SDS/PAGE (10%-acrylamide gel), or applied to a Zorbax GF-250 h.p.l.c. column (0.9 cm × 25 cm) equilibrated with 200 mM-sodium phosphate/0.5 mM-DTT/0.005% NaN_3 at pH 7.0 and with thyroglobulin, catalase, aldolase, BSA and myoglobin as calibration standards. The elution was monitored by the A_{280}. Fractions were collected every 4 s and assayed for PFK-2 activity.

Protein kinase C was purified from bovine brain after chromatography on DEAE-cellulose DE52, phenyl-Sepharose and protamine–agarose as described elsewhere [20,21]. The specific activity of this preparation was 0.8 μmol of [^{32}P]phosphate incorporated into histone H1/min per mg of protein.

Assay of enzyme activities

Purified PFK-2 activity was measured as described by Rider *et al.* [18]. The 'active' form of PFK-2 was measured at pH 6.6 in the presence of 1 mM-fructose 6-phosphate. The total activity was measured at pH 8.5 with 5 mM-fructose 6-phosphate [22]. The sensitivity of PFK-2 activity to inhibition by *sn*-glycerol 3-phosphate was determined at pH 7.1 in the presence of 0.1 mM-fructose 6-phosphate and 0.5 mM-MgATP [18]. The affinity of PFK-2 for fructose 6-phosphate was determined at pH 7.1 in the presence of 5 mM-MgATP. The affinity of MgATP was measured at pH 7.1 by using 1 mM-fructose 6-phosphate.

Protein phosphorylation

PFK-2 (50 μg) was incubated at 30 °C for 30 min in a final volume of 0.25 ml containing 100 mM-KCl, 5 mM-$MgCl_2$, 1 mM-DTT, 1 mM-potassium phosphate, 5 mM-MgATP and 20 mM-Hepes at pH 7.4 with 0.1 m-unit of catalytic subunit of cyclic AMP-dependent protein kinase. Incubations with protein kinase C (0.1 m-units) included 40 μg of phosphatidylserine/ml and 0.2 mM-$CaCl_2$. Samples (20 μl) were used to assay PFK-2 activity at pH 6.6 and 8.5 as previously indicated. One unit of catalytic subunit of cyclic AMP-dependent protein kinase was defined as the activity that incorporates 1 μmol of phosphate into histone H2A/min at 30 °C. One unit of protein kinase C was defined as the amount of enzyme that incorporates 1 μmol of phosphate into histone H1/min at 30 °C.

When the incorporation of [^{32}P]phosphate into the enzyme was measured, purified PFK-2 both from adult and foetal rat liver (100 μg) was incubated at 30 °C for 30 min with 0.1 m-unit of catalytic subunit of cyclic AMP-dependent protein kinase in an assay mixture containing 50 mM-Tris/phosphate, 0.1 mM-EDTA, 2 mM-$MgCl_2$ and 2 mM-DTT at pH 7.4 (final volume of 0.2 ml). Reactions were initiated with 200 μM-[γ-^{32}P]ATP (700 c.p.m./pmol) and samples (20 μl) were collected at the indicated times for measurement of [^{32}P]phosphate incorporation into trichloroacetic acid-insoluble material [18]. After incubation for 30 min, the remaining reaction mixture was denatured with 20 μl of Laemmli [19] sample buffer and used for SDS/PAGE. Incubations with protein kinase C (0.1 m-unit) were carried out in an assay containing 50 mM-Hepes, pH 7.4, 15 mM-β-mercaptoethanol, 0.25 mM-$CaCl_2$, 40 μg of phosphatidylserine/ml, 8 mM-magnesium acetate and 200 μM-[γ-^{32}P]ATP (700 c.p.m./pmol) in a total volume of 0.2 ml. Proteins were detected with Coomassie Blue staining before gel drying and autoradiography.

The endogeneous phosphoenzyme present in purified PFK-2 was determined after incubation of the enzyme (100 μg) with immobilized alkaline phosphatase–agarose (2 units; one unit hydrolysed 1 μmol of *p*-nitrophenyl phosphate/min at pH 10) for 30 min at 30 °C in 20 mM-Tris, pH 8.9, followed by the phosphorylation with the appropriated protein kinase. The percentage of phosphoenzyme was calculated by subtracting the radioactivity incorporated after treatments with alkaline phosphatase from that of untreated samples.

Antibodies against adult rat liver PFK-2/FBPase-2

An antibody specific against a decapeptide corresponding to the *N*-terminus of adult liver PFK-2, previously described [16], was kindly provided by Professor L. Hue and Professor G. G. Rousseau, Lovain Medical School, Brussels, Belgium.

Western-blot analysis

Foetal and adult liver purified PFK-2 were electrophoretically separated in SDS/PAGE as described by Laemmli [19]. After the run, the gel was equilibrated in transfer buffer [25 mM-Tris, 192 mM-glycine, 20% methanol) and proteins were electrophoretically transferred (0.5 mA/cm^2, 15 h) to nitrocellulose membranes [23]. The antigen (PFK-2) was detected by a specific primary antibody. Bound antibody was detected by a peroxidase/anti-peroxidase complex revealed by oxidation of 4-chloro-1-naphthol to a violet product. Quantification of the peroxidase reaction was performed in a densitometer (E-C Apparatus Corp.).

Thiol modification of PFK-2 activity

The purified enzymes (50 μg) were reduced with fresh DTT (2 mM final concn.) and applied to a h.p.l.c. Zorbax GF-250 column equilibrated with 50 mM-sodium phosphate (pH 7.1) to remove the thiol protectants and were immediately alkylated with a fresh solution of 1.5 mM-iodoacetamide. At the indicated times, samples (20 μl) were mixed with 80 μl of 50 mM-DTT in order to stop the alkylation and maintained at 4 °C. The mixture was assayed for PFK-2 activity at 5 mM- or 50 μM-fructose 6-phosphate in the presence of 5 mM-MgATP and 5 mM-phosphate, as previously described.

CNBr cleavage of PFK-2/FBPase-2

The purified enzymes (250 μg) were reduced with fresh DTT (2 mM final concn.) and, after removal of the thiol protectants by h.p.l.c. gel filtration, the proteins were immediately alkylated with a fresh solution of 1.5 mM-iodo[1-^{14}C]acetamide (20 d.p.m./pmol) at 30 °C for 1 h. At the indicated times, samples (10 μl) were stopped with 100 μl of 100 mM-DTT, followed by addition of 1 ml of ice-cold trichloroacetic acid (5%, w/v). After filtration

on GF/C filters (Whatman) the radioactivity was measured. At the end of the incubation period the remaining reaction was stopped with 200 μl of 100 mM-DTT and 1 ml of ice-cold 10% trichloroacetic acid and centrifuged in a Eppendorf Microfuge for 15 min. The pellet was resuspended in 1 vol. (original) of 70% (v/v) formic acid and solid CNBr was added to a final concentration of 2 mg/ml. The tube was flushed with N_2 and incubated for 24 h at room temperature. After vacuum drying of the tubes, the peptides were resuspended in 100 μl of the medium used for the resolution from the h.p.l.c. column (see below). A similar protocol, but in the presence of 1 mM-DTT, was used for CNBr digestion of PFK-2 after phosphorylation either by the catalytic subunit of the cyclic AMP-dependent protein kinase or by protein kinase C as previously described. The solution containing the CNBr fragments was applied to a Brownlee (25 cm × 0.46 cm) reverse-phase column (Spheri-5 RP8 5 μM) and resolved by an isocratic gradient of 30% acetonitrile/70% of a mixture of 0.6% acetic acid and 0.003% triethylamine at a flow rate of 1 ml/min, by using a h.p.l.c. system. Injection of mixtures containing foetal and adult peptides were used to assess the accuracy of the column resolution. The eluted peptides were detected at 280 nm and by counting the emerging radioactivity.

Protein concentration was determined by the Bradford method [24], with bovine serum albumin as a standard.

RESULTS

Enzyme characterization

PFK-2 from foetal and adult rat liver was purified by poly(ethylene glycol) fractionation, DEAE-cellulose DE52 and Blue Sepharose chromatography followed by gel filtration on a Zorbax GF-250 h.p.l.c. column. The physical behaviour of both enzymes throughout the purification procedure was very similar, except for the small difference in salt concentration needed to elute the enzyme from the DEAE-cellulose DE52 column (300 mM- and 375 mM-KCl for adult and foetal enzyme respectively). The native molecular masses determined for the foetal and adult enzymes were 89.1 and 100.0 kDa respectively (Fig. 1*a*). Gel filtration on Sephacryl S-200 gave the same results. However, the subunit molecular mass deduced from the relative mobility on SDS/PAGE (10%-acrylamide gels) was 55 kDa for both enzymes (Fig. 1*b*). The more relevant kinetic properties of purified foetal and adult PFK-2 are shown in Table 1. No significant differences between the enzymes were observed in the affinity for fructose 6-phosphate and ATP. However, in agreement with previous work [6], a marked difference was observed in the inhibition by *sn*-glycerol 3-phosphate; the apparent K_i values were 2.7 ± 0.3 and 0.15 ± 0.02 mM respectively for foetal and adult PFK-2. The specific activities of both enzymes were 80 ± 11 and 55 ± 9 m-units/mg of protein for foetal and adult PFK-2.

PFK-2 phosphorylation

To analyse further the nature of the foetal and adult PFK-2, the purified enzymes were incubated with immobilized alkaline phosphatase before being assayed as substrates of the catalytic subunit of the cyclic AMP-dependent protein kinase and of protein kinase C. In addition to this, the effect of this phosphorylation on the kinetic properties of both enzymes was measured. As shown in Fig. 2(*a*), incubation of foetal liver PFK-2 with cyclic AMP-dependent protein kinase resulted in the phosphorylation of the enzyme, but this covalent modification was not followed by changes either in the affinity for fructose 6-phosphate (results not shown) or in the ratio between the active and total enzyme determined by the relative activities at pH 8.5 and pH 6.6 (1.2 and 0.8 for the phospho- and dephospho-enzyme

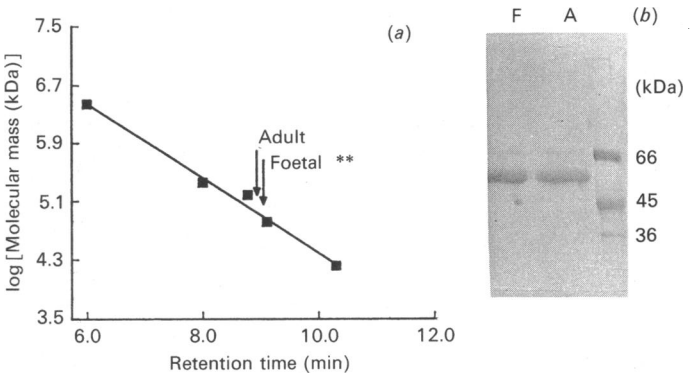

Fig. 1. Determination of the molecular mass of PFK-2

(*a*) Samples of foetal or adult PFK-2 (25 μg) were injected into a h.p.l.c. Zorbax GF-250 column. The A_{280} was recorded and fractions (4s) were collected to assay PFK-2 activity. The calibration standards were thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa) and myoglobin (17 kDa). Results are expressed as means ± S.E.M. of the retention times at peak values for 5–7 different injections: ** significantly different from adult enzyme ($P < 0.05$). (*b*) SDS/PAGE (10%-acrylamide gel) analysis of foetal (F) and adult (A) PFK-2.

respectively). As has been well established [25], the adult liver enzyme processed in parallel with the foetal one was also phosphorylated by the cyclic AMP-dependent protein kinase, but exhibited kinetic changes in the activity ratio at pH 8.5/pH 6.6 (1.2 and 3.5 for the dephospho- and phospho-enzyme respectively; results not shown). In addition to the cyclic AMP-dependent protein kinase, foetal PFK-2 was also incubated with purified bovine brain protein kinase C. As shown in Fig. 2(*a*), the foetal enzyme was phosphorylated by protein kinase C. However, the phosphoenzyme did not show changes in the activity ratio at pH 8.5 and pH 6.6. The stoichiometry of the phosphorylation of foetal PFK-2 by cyclic AMP-dependent protein kinase and protien kinase C was 0.95 mol of [^{32}P]phosphate/mol of enzyme subunit (Fig. 2*b*). Consistent with previous work [9], the adult enzyme was not phosphorylated by protein kinase C (results not shown).

When the foetal enzyme was incubated with protein kinase C after phosphorylation with cyclic AMP-dependent protein kinase, an additional 0.9 mol of phosphate was incorporated per mol of subunit, suggesting the presence of two different phosphorylation sites per enzyme subunit (see Fig. 6). Foetal PFK-2 phosphorylated by both kinases did not exhibit kinetic changes in the affinity for fructose 6-phosphate or in the maximal activity.

Table 1. Summary of kinetic properties of purified foetal and adult rat liver PFK-2

The enzyme activity was assayed at pH 7.1 in the presence of 1 mM-potassium phosphate. The inhibition by *sn*-glycerol 3-phosphate was measured with 0.1 mM-fructose 6-phosphate and 0.5 mM-MgATP. Results are means ± S.E.M. of duplicates from three different enzyme purifications.

	Foetal	Adult
K_m for fructose 6-phosphate (μM)	44 ± 6	47 ± 7
K_m for ATP (μM)	195 ± 30	190 ± 23
K_i for *sn*-glycerol 3-phosphate (mM)	> 2.7	0.15 ± 0.02
Sp. activity (m-units/mg of protein)	80 ± 11	55 ± 9

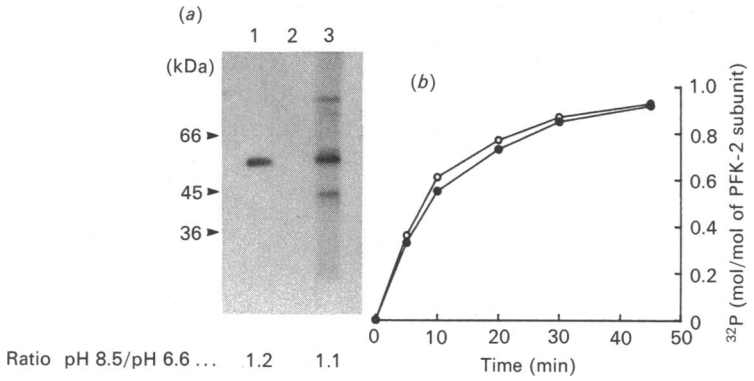

Fig. 2. Phosphorylation of PFK-2 by the catalytic subunit of the cyclic AMP-dependent protein kinase and by protein kinase C

(*a*) Purified foetal PFK-2 (100 μg) was incubated in parallel either with 0.1 m-unit of cyclic AMP-dependent protein kinase or 0.1 m-unit of protein kinase C for 30 min at 30 °C in the presence of 200 μM-[γ-^{32}P]ATP (700 c.p.m./pmol). Samples were collected to determine the activity ratio at pH 8.5/pH 6.6, the stoichiometry of the reaction and the identity of the phosphoenzyme. (*a*) Autoradiography of foetal PFK-2 phosphorylation: lane 1, PFK-2 plus 0.1 m-unit of cylic AMP-dependent protein kinase; lane 2, PFK-2 in the absence of protein kinase; lane 3, PFK-2 plus protein kinase C in the presence of Ca^{2+} and phospholipids. (*b*) Time course of phosphorylation of foetal liver PFK-2 by cyclic AMP-dependent protein kinase (●) and by protein kinase C (○).

Treatment of adult and foetal PFK-2 with alkaline phosphatase–agarose before phosphorylation with protein kinases showed that the amount of phosphoenzyme in the purified PFK-2 was about 10–12% when phosphorylated by cyclic AMP-dependent protein kinase, whereas no changes were observed in the case of phosphorylation of the foetal enzyme by protein kinase C.

Western-blot analysis of PFK-2

The differences in the response to cyclic AMP-dependent protein kinase and protein kinase C by both enzymes suggested the existence of two isoenzymes in foetal and adult liver. As shown in Fig. 3, Western-blot analysis of purified foetal and adult PFK-2 using a specific antibody against a decapeptide corresponding to the *N*-terminus of the adult liver enzyme revealed a different recognition pattern for both proteins. The staining of the adult enzyme is about 6-fold higher than for the foetal protein, suggesting a different *N*-terminal sequence for each protein.

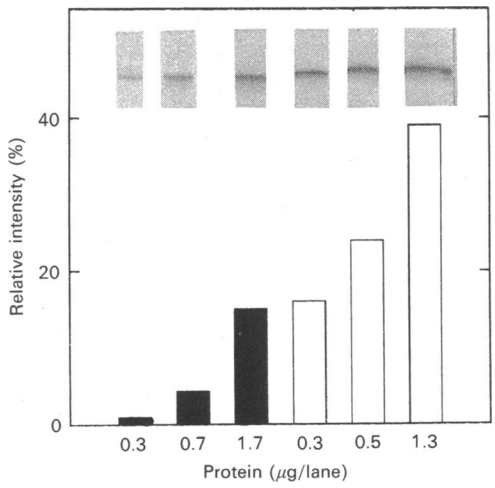

Fig. 3. PFK-2 characterization by a liver-type specific antibody

Purified foetal (■) and adult (□) PFK-2 were submitted to Western-blot analysis and recognition by an antibody raised against a decapeptide specific for the adult liver enzyme. The peroxidase-coupled reaction was quantified in a densitometer and the relative colour intensities were expressed as percentages of the total signal.

Effect of thiol-group modification on PFK-2 activity

Iodoacetamide and related thiol-modifying reagents have important effects on PFK-2 activity [26,27]. Alkylation of purified adult liver PFK-2 with iodoacetamide produced a 10–20-fold decrease in the affinity for fructose 6-phosphate, and a 10-fold increase in the $V_{max.}$ of the enzyme [27]. To study the effect of alkylation on PFK-2 activity, both foetal and adult liver enzymes were incubated with 1.5 mM-iodoacetamide and samples were collected and assayed for PFK-2 activity at saturating and subsaturating concentrations of fructose 6-phosphate. As shown in Fig. 4(*a*), a 4-fold increase in $V_{max.}$ of the adult enzyme was observed, whereas only a 2-fold increase was evidenced for the foetal enzyme. In both cases, a clear and parallel decrease in the affinity for fructose 6-phosphate was observed.

The time course of incorporation of iodo[1-^{14}C]acetamide into foetal and adult PFK-2 is shown in Fig. 4(*b*). In both cases a maximum of 3–4 readily accessible cysteine residues per molecule of PFK-2 subunit may be alkylated. The apparent correlation between the mol of acetamide incorporated/mol of enzyme subunit and the activation of the kinase for the adult enzyme should be noted. However, although the kinetics of the incorporation of acetamide into PFK-2 is similar for both enzymes, the maximal activation of the foetal enzyme is obtained at lower stoichiometries of alkylation than for the adult enzyme (Fig. 4).

CNBr peptides of PFK-2

To obtain further structural information about the foetal and adult PFK-2, the purified enzymes were alkylated with iodo[1-^{14}C]acetamide, proteolysed with CNBr, and the resulting peptides were analysed on a h.p.l.c. reverse-phase column. As shown in Fig. 5, only one [^{14}C]carboxyamidomethylated fragment was evident for adult PFK-2, whereas two labelled peptides were observed for the foetal enzyme. From the specific radioactivity of iodoacetamide and the analysis of the relative intensity of the radiolabelled peaks, it can be calculated that for the foetal enzyme the relative number of cysteine residues present in the peptides eluted at 3.8 and 8.2 min would be 1.1 and 2.5, whereas for the adult enzyme the peak eluted at 8.6 min corresponds to 3 residues.

In addition to alkylation, foetal and adult PFK-2 were

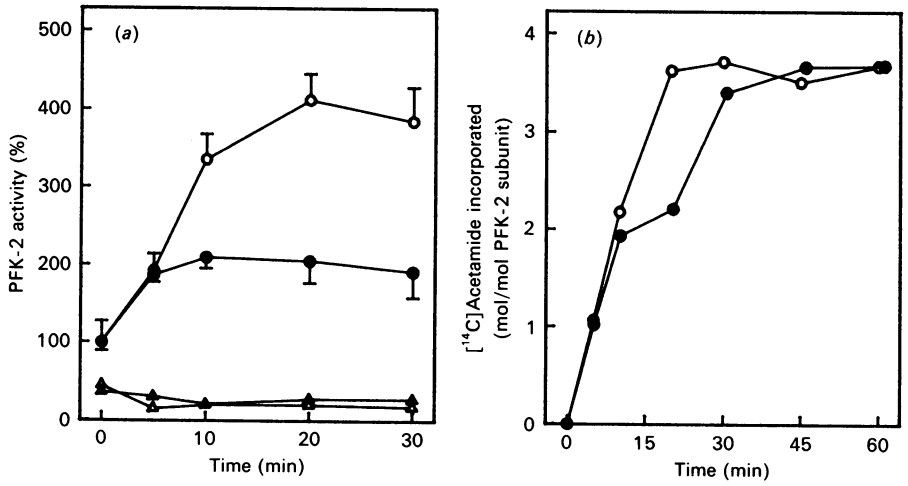

Fig. 4. Time course of PFK-2 alkylation

Foetal (●, ▲) and adult (○, △) PFK-2 were processed by h.p.l.c. gel filtration to remove DTT, and were immediately alkylated with 1.5 mM-iodoacetamide. (*a*) Effect of alkylation on PFK-2 activity assayed with 5 mM- (●, ○) or 50 μM- (▲, △) fructose 6-phosphate. (*b*) Incorporation of iodo[1-^{14}C]acetamide into foetal (●) and adult (○) PFK-2. Results are means ± S.E.M. of three different assays (*a*) or means of duplicate (*b*).

phosphorylated by the catalytic subunit of the cyclic AMP-dependent protein kinase in the presence of [γ-^{32}P]ATP, followed by digestion with CNBr. Fig. 6(*a*) shows the elution profile of the CNBr peptides on a h.p.l.c. reverse-phase column for both proteins. As expected from the stoichiometry of the phosphorylation reaction, only one [^{32}P]phosphate-labelled peak was evident for each protein; however, the phosphopeptides emerged with different retention times (3.24 and 4.0 min for adult and foetal peptides respectively), indicating that the phosphorylation sites were located in two different peptides.

When samples of foetal PFK-2 phosphorylated by cyclic AMP-dependent protein kinase were submitted to phosphorylation by protein kinase C and digested with CNBr, two phosphopeptides were evident (retention times 4.0 and 6.3 min respect-

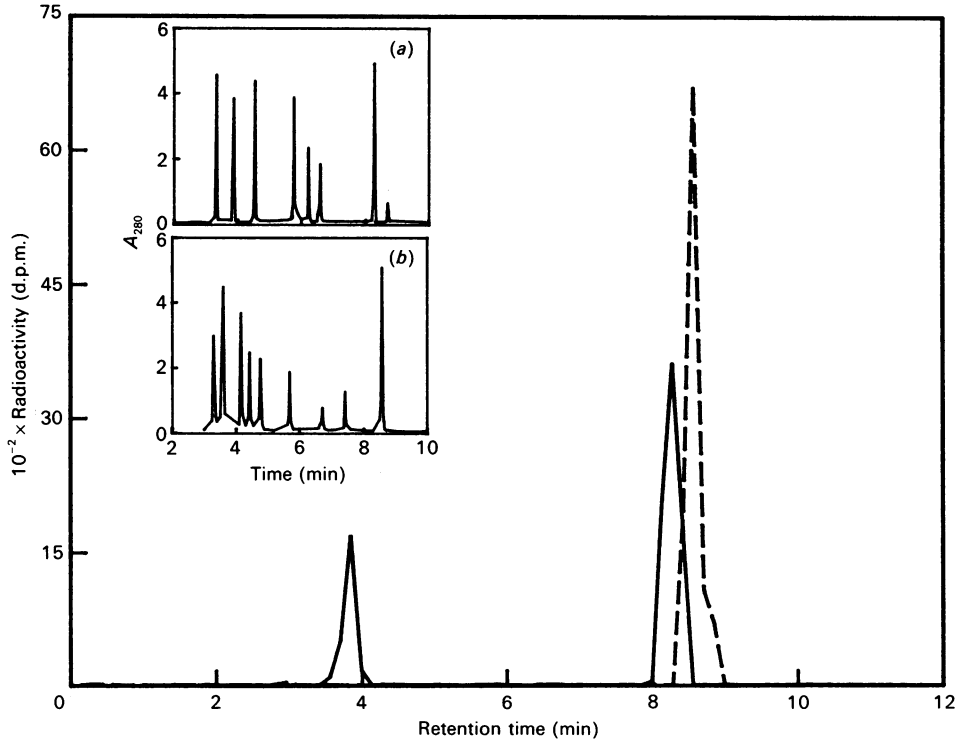

Fig. 5. Identification of carboxyamidomethylated peptides of PFK-2 after CNBr proteolysis

Foetal (——) and adult (– – – –) PFK-2 (250 μg) were reduced with 2 mM-DTT, and processed through a h.p.l.c. Zorbax GF-250 gel-filtration column to remove the remaining DTT, followed by incubation with 1.5 mM-iodo[1-^{14}C]acetamide. After digestion with CNBr in 70% (v/v) formic acid and vacuum drying, samples were resuspended in h.p.l.c. elution medium and injected (25 μl) into a h.p.l.c. reverse-phase column and the radioactivity was measured. Insets show the peptide elution profile of foetal (*a*) and adult (*b*) PFK-2.

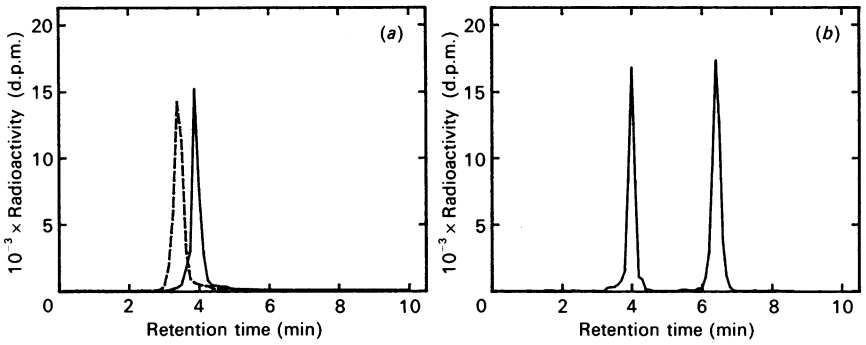

Fig. 6. Identification of phosphopeptides of PFK-2 after CNBr proteolysis

(*a*) Foetal (——) and adult (– – – –) PFK-2 were incubated in the presence of 0.1 m-unit of the catalytic subunit of the cyclic AMP-dependent protein kinase, followed by digestion with CNBr in 70% formic acid. Samples (25 μl) were injected into a h.p.l.c. reverse-phase column and the radioactivity was measured. (*b*) Foetal PFK-2 was phosphorylated by protein kinase C after incubation for 30 min with cyclic AMP-dependent protein kinase, followed by digestion with CNBr. Samples were injected into a h.p.l.c. reverse-phase column and the radioactivity was measured.

ively), indicating the presence of two different phosphorylation sites in the protein (Fig. 6*b*).

DISCUSSION

This report presents further evidence suggesting that PFK-2/FBPase-2 from foetal rat liver is different from the adult enzyme. The purification procedure described for the liver enzyme was suitable for the purification of foetal PFK-2. The specific activity of the foetal enzyme was about 1.5 times that for the adult enzyme. However, the FBPase-2 activity of the foetal enzyme was lower than 0.4 m-unit/mg of protein, in contrast with the adult tissue, which contained 10.1 m-units/mg of protein [6]. The molecular mass of the foetal liver PFK-2 determined by gel filtration in h.p.l.c. was smaller than for the adult enzyme (89.1 and 100 kDa respectively), whereas SDS/PAGE gave a subunit molecular mass of 55 kDa without difference between both enzymes. In agreement with this observation, mixtures of foetal and adult enzyme exhibited only one band of 55 kDa. The discrepancies between these results are unclear, but are probably related to the three-dimensional structure of the enzymes. In this sense, it is important to note the unusual behaviour of the enzyme in ultrafiltration through 30 kDa exclusion membranes, from which foetal PFK-2 was not retained. Several kinetic properties of purified foetal and adult liver PFK-2 were compared. The affinities for its two substrates were very similar for both enzymes. However, foetal liver PFK-2 was about 16-fold less sensitive than the adult enzyme to inhibition by *sn*-glycerol 3-phosphate.

Adult liver PFK-2 was inactivated upon incubation with MgATP and the cyclic AMP-dependent protein kinase [28] (ratios pH 8.5/pH 6.6 were 1.2 and 3.5 for the dephospho- and phospho-enzyme respectively). The stoichiometry of the phosphate incorporation was approx. 1. Foetal liver PFK-2 was also phosphorylated by cyclic AMP-dependent protein kinase, exhibiting a stoichiometry of 0.95, but, in contrast with the adult enzyme, there were no changes in its kinetic properties. In both cases only one phosphopeptide was evident after phosphorylation of each enzyme and CNBr digestion. Adult liver was not phosphorylated by protein kinase C as previously described [9]; by contrast, foetal liver PFK-2 was phosphorylated by protein kinase C with a stoichiometry of 0.95, but the activity remained unaffected. In this respect, the foetal fat liver PFK-2 resembles the myocardial PFK-2, which is phosphorylated by either cyclic AMP-dependent protein kinase or protein kinase C [10], although in that report phosphorylation of PFK-2 increased the affinity for fructose 6-phosphate and the $V_{max.}$. Moreover, when foetal PFK-2 was sequentially phosphorylated by cyclic AMP-dependent protein kinase and protein kinase C, no changes were observed in the kinetic behaviour of the enzyme.

Western-blot analysis of purified PFK-2 from foetal or adult liver using a specific antibody raised against the *N*-terminus of the adult liver PFK-2 clearly showed that both proteins differ at least in the *N*-terminus domain. The staining of the adult enzyme was at least 6 times that of the foetal protein (Fig. 3). It has been described [10,11,29] that forms of PFK-2 present in other tissues, such as heart, muscle and HTC cells, basically differ in the *N*-terminus of the protein. The rat skeletal-muscle enzyme is very similar in structure to the rat liver enzyme, except for the lack of one peptide (corresponding to nucleotides 14–28) and the lack of a phosphorylation site by the cyclic AMP-dependent protein kinase. cDNA analysis of HTC cells predicts a protein in which the first 32 residues of liver PFK-2 including the cyclic AMP target sequence have been replaced by a unique *N*-terminal decapeptide. Furthermore, the phosphorylation site of the myocardial PFK-2 by cyclic AMP-dependent protein kinase is clearly different from Ser^{32}, as occurs in the liver enzyme.

Rat liver PFK-2/FBPase-2 contains 10 cysteine residues/subunit [26]. Three to four of the thiol groups are essential for catalysis in the kinase reaction [26,27,30,31]. Alkylation of these cysteine residues located near the kinase domain of PFK-2 results in a decrease in its affinity for fructose 6-phosphate and a stimulation in $V_{max.}$. Foetal PFK-2 also contains 3–4 accessible cysteine residues, although the effect of alkylation on the $V_{max.}$ is lower than for the adult enzyme. One possible explanation for this different behaviour could be related to the fact that alkylation inhibits FBPase-2 [27], and this activity is very low in the foetal enzyme. When these reactive cysteine residues were labelled with iodoacetamide and the carboxyamidomethylated enzyme digested with CNBr, and these fragments were analysed by reverse-phase h.p.l.c., only one peptide was labelled in the adult enzyme [27,30]. Study of the primary sequence of the enzyme subunit revealed that these cysteine residues were in the *N*-terminal half of the subunit at residues 107, 160, 183 and 198 [30]. In agreement with these results, CNBr digestion of carboxyamidomethylated adult liver PFK-2 showed nine peptides on monitoring the A_{280}, and only one major labelled peak eluted at 8.6 min, which presumably corresponded to the fragment containing residues 47–277 described by Lively *et al.* [30]. On the contrary, foetal PFK-2 showed after digestion with CNBr eight peptides by monitoring the A_{280}, and two laballed peaks: one eluted at

8.2 min and a specific peptide eluted at 3.8 min. Comparison of the CNBr peaks sugested that the ratio of carboxyamidomethylated residues eluted at 8.6 and 8.3 min was 3:2.6 (adult:foetal peptides), and 1.1 for the specific peak eluted at 3.8 min, for the foetal enzyme. These results suggested that digestion with CNBr produced different peptides in foetal and adult liver PFK-2. In addition to alkylation, foetal and adult PFK-2 were phosphorylated by the cyclic AMP-dependent protein kinase, followed by digestion with CNBr and h.p.l.c. analysis. Previous reports [30] identified the cyclic AMP-dependent phosphorylation site of the adult liver enzyme located in a peptide containing residues 5–43 after treatment with CNBr which corresponds to Ser^{32} of the protein. On the other hand, cyclic AMP-dependent protein kinase and protein kinase C phosphorylated heart PFK-2 in the same CNBr peptide (Arg^{463}–Pro^{479}), at residues Ser^{466} and Thr^{475} respectively, and therefore located in the *C*-terminus region of the protein [15]. Our results showed only one labelled peak for both foetal and adult PFK-2, but the phosphopeptides were eluted with different retention times (3.54 and 3.24 min respectively), indicating that the phosphorylation sites by cyclic AMP-dependent protein kinase were different and located on two different peptides. Moreover, sequential phosphorylation by the cyclic AMP-dependent protein kinase and protein kinase C revealed the presence of two specific phosphorylation sites in the foetal enzyme. All of these results suggest that the foetal enzyme of PFK-2 is distinct from the adult form on the following basis: (*a*) differential behaviour on gel filtration; (*b*) a different peptide profile after CNBr digestion; (*c*) the foetal enzyme is phosphorylated by the cyclic AMP-dependent protein kinase and by protein kinase C at two different sites, although without changes in kinetic properties; and (*d*) a different immunogenic response is observed with antibodies recognizing the *N*-terminal region of the protein.

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